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Technical Advance

Quantitative Gene Expression Analysis in Microdissected Archival Formalin-Fixed and Paraffin-Embedded Tumor Tissue

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Formalin-fixed, paraffin-embedded tissue is the most widely available material for retrospective clinical studies. In combination with the potential of genomics, these tissues represent an invaluable resource for the elucidation of disease mechanisms and validation of differentially expressed genes as novel therapeutic targets or prognostic indicators. We describe here an approach that, in combination with laser-assisted microdissection allows quantitative gene expression analysis in formalin-fixed, paraffin-embedded archival tissue. Using an optimized RNA microscale extraction procedure in conjunction with real-time quantitative reverse transcriptase-polymerase chain reaction based on fluorogenic TaqMan methodology, we analyzed the expression of a panel of cancer-relevant genes, *EGF-R, HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, *MDM2*, and *HPRT* and *PGK* as controls. We demonstrate that expression level determinations from formalin-fixed, paraffin-embedded tissues are accurate and reproducible. Measurements were comparable to those obtained with matching fresh-frozen tissue and neither fixation grade nor time significantly affected the results. Laser microdissection studies with 5-μm thick sections and defined numbers of tumor cells demonstrated that reproducible quantitation of specific mRNAs can be achieved with only 50 cells. We applied our approach to HER-2/neu quantitative gene expression analysis in 54 microdissected tumor and nonneoplastic archival samples from patients with Barrett's esophageal adenocarcinoma and showed that the results matched those obtained in parallel by fluorescence *in situ* hybridization and immunohistochemistry. Thus, the combination of laser-assisted microdissection and real-time TaqMan reverse transcriptase-polymerase chain reaction opens new avenues for the investigation and clinical validation of gene expression changes in archival tissue specimens. *(Am J Pathol 2001, 158:419–429)*

Quantitative determination of gene expression levels is a powerful approach for the comparative analysis of normal and neoplastic tissues. Rapid progress in the human genome project and the development of new techniques such as cDNA array hybridization and serial analysis of gene expression now permit the transcript level analysis of thousands of genes in a single experiment.[1–3] To allow conclusions regarding the clinical significance of the results obtained with such techniques, the examination of large numbers of pathological tissue specimens representing different disease stages and histological tumor types and grades is essential. Archival formalin-fixed, paraffin-embedded (FFPE) tissue specimens, in conjunction with clinical data are the most widely available basis for such retrospective studies. The reliable quantitation of gene expression in formalin-fixed, paraffin-embedded tissue, however, has been subject to serious limitations so far, although previous studies have demonstrated that nucleic acids may be extracted from formalin-fixed, paraffin-embedded material.[4–6] Although this is a lesser problem for DNA, RNA isolated from paraffin-embedded tissue blocks is of poor quality because extensive degradation of RNA can occur before completion of the formalin fixation process.[7] Moreover, formalin fixation causes cross-linkage between nucleic acids and proteins and covalently modifies RNA by the addition of mono-methylol groups to the bases, making subsequent RNA extraction, reverse transcription and quantitation analysis problematic.[8]

Real-time quantitative TaqMan reverse transcriptase-polymerase chain reaction (QRT-PCR) analysis has re-

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cently been introduced as a sensitive, accurate, and highly reproducible method to study gene expression. The technique is based on the 5′ nuclease activity of *Taq* DNA polymerase and involves cleavage of a specific fluorogenic hybridization probe that is flanked by PCR primers spanning an amplicon range of 60 to 150 bp.[9,10] Because of the small target size, this approach seemed to be particularly suitable for quantitative determination of gene transcript levels even in tissue extracts containing partially fragmented RNA. As this experimental strategy requires only minute amounts of RNA it should be applicable to small clinical biopsies and microdissected cell clusters from frozen or formalin-fixed, paraffin-embedded tissue sections. Laser-assisted microdissection has become indispensable for the selective analysis of stroma-free tumor cell populations circumventing the problem of tissue heterogeneity as well as providing the possibility to assign characteristic gene expression patterns to particular histological phenotypes.[11,12]

Here we present a new approach toward the detection and quantitation of specific mRNA levels in formalin-fixed, paraffin-embedded samples that combines real-time QRT-PCR and laser-assisted microdissection. Using a panel of cancer-relevant genes, we performed quantitative gene expression analysis in matched frozen and formalin-fixed, paraffin-embedded tissue samples. We examined the influence of different parameters on reliable and reproducible mRNA quantitation, including several microscale RNA extraction protocols, formalin-fixation time and laser microdissection of defined numbers of cells. Finally, we analyzed 54 microdissected nonneoplastic and neoplastic samples from 26 archival adenocarcinoma of the esophagus by QRT-PCR for HER-2/*neu* gene expression and compared the results with those obtained by fluorescence *in situ* hybridization (FISH) and immunohistochemistry.

Materials and Methods

Cell Lines

Human A431 epidermoid carcinoma cells (CRL-1555; American Type Culture Collection, Rockville, MD) were grown in Dulbecco's minimal essential medium supplemented with 2 mmol/L L-glutamine and 10% fetal calf serum. Human HT29 colon adenocarcinoma cells (HTB38; American Type Culture Collection) were grown in McCoy's medium supplemented with 2 mmol/L glutamine and 10% fetal calf serum. Media and supplements were purchased from Gibco BRL (Eggenstein, Germany).

Tumor Formation in Nude Mice

Five-week-old athymic (*nu/nu*) mice were obtained from Charles River Breeding Laboratories (Wilmington, MA). Cultured A431 and HT29 tumor cells were resuspended in 100 μl of sterile phosphate-buffered saline at a cell density of 2×10^6 and injected subcutaneously into the flank region of nude mice. Tumor formation was monitored twice weekly by measuring the width and length of the tumors. Animals with mean tumor diameters of 15 mm were sacrificed and tumor samples were cut in halves. Half of each of the tumors was fixed in 10% buffered formalin for 24 hours within 1 hour after surgical removal and paraffin-embedded using standard procedures, the other half of the tumor was immediately snap-frozen and stored in liquid nitrogen until use.

Tissue Samples

For testing the specificity of the QRT-PCR and variables of the formalin fixation procedure, liver, uterus with leiomyoma, and a prostate cancer specimen obtained from three different patients were used. The tissue samples were fixed in 10% neutral-buffered formalin for 20 hours within 1 hour after surgical removal and paraffin-embedded using standard procedures. For the HER-2/*neu* analyses, archival material from formalin-fixed, paraffin-embedded tissue obtained from 26 patients with primary Barrett's adenocarcinoma of the distal esophagus was used. Serial sections were cut for immunohistochemistry (5 μm), FISH (10 μm), and quantitative real-time RT-PCR analysis (5 μm). Corresponding areas on sequential sections were investigated by the three methods.

Tissue Preparation and Microdissection

Using RNase-free conditions, frozen tissue blocks were sectioned at 5 μm in a cryostat, mounted on noncoated clean glass slides, and stored at −80°C until use. Formalin-fixed, paraffin-embedded tissue samples were cut in 5-μm-thick sections on a microtome with a disposable blade. For microdissection, sections were deparaffinized in two changes of xylene for 10 minutes, rehydrated in 100% ethanol, 90% ethanol, and 70% ethanol for 5 minutes each, stained with hematoxylin and eosin (H&E) for 45 seconds, rinsed in RNase-free H_2O for 30 seconds, and finally immersed in 100% ethanol for 1 minute. The PALM Laser-MicroBeam System (P.A.L.M., Wolfratshausen, Germany) was used for microdissection. This system consists of a 337-nm pulsed nitrogen laser coupled to an inverted microscope via the epifluorescence illumination path. After selecting the cells of interest, adjacent cells were photolysed by the microbeam. To retrieve the selected cells from the slide, a computer-controlled micromanipulator and conventional sterile needles were used to pick and transfer the cells into a reaction tube as described previously.[11] For quantitative gene expression analyses in formalin-fixed, paraffin-embedded A431 and HT29 xenografts, groups of tumor cells (n_{cell} = ~10,000, ~1,000, 100, and 50) were laser-microdissected from H&E-stained 5-μm sections. For the HER-2/*neu* gene expression analyses in Barrett's adenocarcinoma, groups of ~1,000 cells were microdissected from nonneoplastic squamous epithelium or gastric mucosa and carcinoma lesions, respectively.

Extraction of Total RNA from Formalin-Fixed, Paraffin-Embedded Tissue

Total RNA from liver, uterus, leiomyoma, the prostate cancer specimen, and A431 and HT29 tumor xenografts was extracted from formalin-fixed, paraffin-embedded tissue using a modification of the method described by Rupp and Locker.[6] Briefly, for the analysis of unstained 5-μm sections, tissue was scraped off and paraffin was removed by extracting two times with 1 ml of xylene for 10 minutes followed by rehydration through subsequent washes with 100, 90, and 70% ethanol diluted in RNase-free water. After each step, the tissue was collected by centrifugation at 16,000 $\times$ *g* for 5 minutes. After the final 70% ethanol wash, the pellet was dried, resuspended in 200 μl of RNA lysis buffer containing 10 mmol/L Tris/HCL (pH 8.0), 0.1 mmol/L ethylenediaminetetraacetic acid (pH 8.0), 2% sodium dodecyl sulfate (pH 7.3), and 500 μg/ml proteinase K (Sigma, Deisenhafen, Germany) and incubated at 60°C for 16 hours until the tissue was completely solubilized. Alternatively, for the microdissection experiments with A431 and HT29 tumor xenografts and for the HER-2/*neu* gene expression analyses in Barrett's adenocarcinoma, microdissected cells from H&E-stained sections were directly transferred into a sterile 1.5-ml tube and lysed in 200 μl of RNA lysis buffer. RNA was purified by phenol and chloroform extractions followed by precipitation with an equal volume of isopropanol in the presence of 0.1 volume of 3 mol/L sodium acetate (pH 4.0), and 1 μl of 10 mg/ml of carrier glycogen at −20°C. The RNA pellet was washed once in 70% ethanol, dried, and resuspended in 10 μl of RNase-free water. In comparing experiments, RNA was isolated from formalin-fixed, paraffin-embedded liver tissue using either the method described above with Proteinase K digestion at 60°C and 42°C or three different protocols[13–15] after tissue sections had been deparaffinized as described above.

Extraction of RNA from Frozen Tissue

Total RNA was extracted from scraped frozen tissue sections with the Micro RNA Isolation Kit (Stratagene, San Diego, CA) following the manufacturer's protocol.

Reverse Transcription

RNA extracted from frozen and formalin-fixed, paraffin-embedded tissue sections was reverse-transcribed in a final volume of 20 μl using M-MLV reverse transcriptase (Gibco-BRL) in the manufacturer's buffer containing 1 mmol/L dNTPs, 40 U of RNase inhibitor (Amersham Pharmacia Biotech, Freiburg, Germany), 300 ng of random hexamers (Pharmacia), and 7 μl of RNA. The reactions took place at 42°C for 60 minutes, followed by 95°C for 5 minutes and 4°C for 5 minutes.

Real-Time Quantitative RT-PCR

Real-time quantitative RT-PCR analyses for *EGF-R, HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, *MDM1*, *HPRT*, and *PGK* mRNAs were performed using the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc., Foster City, CA). Intron-spanning primers and probes for the TaqMan system were designed to meet specific criteria by using Primer Express software (Perkin Elmer, Foster City, CA) and were synthesized by PE ABI (Weiterstadt, Germany). The 5′- and 3′-end nucleotides of the probe were labeled with a reporter (FAM = 6-carboxy-fluorescein) and a quencher dye (TAMRA = 6-carboxy-tetramethylrhodamine). The sequences of the PCR primer pairs and fluorogenic probes that were used for each gene are shown in Table 1. The oligonucleotides are designated by the nucleotide position relative to *EGF-R* GenBank accession no. X00588, *HER-2/neu* GenBank accession no. M11730, *FGF-R4* GenBank accession no. L03840, *p21/WAF1/Cip1* GenBank accession no. L25610, *MDM2* GenBank accession no. M92424, *HPRT* GenBank accession no. M31642, *TBP* (a component of the DNA-binding protein complex TFIID) GenBank accession no. X54993 and *PSA* (prostate-specific antigen) GenBank no. X05332. *PGK* primers and probes were purchased from Perkin-Elmer. The principle of real-time RT-PCR has been described in detail elsewhere.[9,10] Briefly, real-time RT-PCR is based on fluorescence emission by a sequence-specific, nonextendable probe labeled with a 5′-reporter and 3′-quencher dye. During the extension phase of the PCR, the nucleolytic activity of the *Taq* DNA polymerase cleaves the hybridization probe and the subsequent separation of quencher and reporter dye releases a fluorescence signal that is monitored every 8.5 seconds by a sequence detector. The signal is normalized to an internal reference (ΔRn) and the software sets the threshold cycle Ct, when ΔRn becomes equal to 10 standard deviations of the baseline. Ct is used for quantitation of the input target number. The relative expression level of the gene of interest was computed with respect to the internal standard, PGK to normalize for variances in the quality of RNA and the amount of input cDNA. For each experimental sample, the amount of target and endogenous reference was determined from a standard curve. The latter was constructed with fivefold serial dilutions of A431 carcinoma cell line cDNA (100,000 pg to 16 pg) and was run in duplicate during every experiment. The amount of target gene was divided by the endogenous reference amount to obtain a normalized target value. PCR was performed with the TaqMan Universal PCR Master Mix (PE, Applied Biosystems) using 3 to 5 μl of diluted cDNA, 200 nmol/L of the probe, and 300 nmol/L primers (except HER-2/*neu* RP and FGF-R4 FP, which were used at 50 nmol/L and 900 nmol/L, respectively) in a 30-μl final reaction mixture. After a 2-minute incubation at 50°C to allow for UNG cleavage, AmpliTaq Gold was activated by an incubation for 10 minutes at 95°C. Each of the 50 PCR cycles consisted of 15 seconds of denaturation at 95°C and hybridization of probe and primers for 1 minute at 60°C.

Table 1. Sequence of TaqMan Primers and Probes Used in This Study

Oligonucleotide	Location	Sequence	Size PCR product
EGF-R FP	1156F	5′-CGCAAGTGTAAGAAGTGCGAA-3′	93 bp
EGF-R RP	1248R	5′-CGTAGCATTTATGGAGAGTGAGTCT-3′	
EGF-R Probe	1180	5′-CCTTGCCGCAAAGTGTGTAACGGAAT-3′	
HER-2/neu FP	2607F	5′-CCAGGACCTGCTGAACTGGT-3′	72 bp
HER-2/neu RP	2678R	5′-TGTACGAGCCGCACATCC-3′	
HER-2/neu Probe	2632	5′CAGATTGCCAAGGGGATGAGCTACCTG-3′	
FGF-R4 FP	715F	5′-TCCGCTGGCTTAAGGATGG-3′	86 bp
FGF-R4 RP	800R	5′-CACGAGACTCCAGTGCTGATG-3′	
FGF-R4 Probe	753	5′-AACCGCATTGGAGGCATTCGGC-3′	
p21 FP	479F	5′-CTGGAGACTCTCAGGGTCGAA-3′	66 bp
p21 RP	544	5′-GGCGTTTGGAGTGGTAGAAATCT-3′	
p21 Probe	501	5′-ACGGCGGCAGACCAGCATGA-3′	
p21 RPI	576R	5′-GGATTAGGGCTTCCTCTTGGA-3′	98 bp
p21 RPII	600R	5′-CAGGACTGCAGGCTTCCTGT-3′	122 bp
p21 RPIII	636R	5′-AAGATGTAGAGCGGGCCTTTG-3′	158 bp
p21 RPIV	660R	5′-ACACACAAACTGAGACTAAGGCAGA-3′	182 bp
p21 RPV	797R	5′-CCAGCACTCTTAGGAACCTCTCA-3′	319 bp
p21 RPVI	852R	5′-AAAGGAGAACACGGGATGAGG-3′	374 bp
MDM2 FP	270F	5′-CGACTCCAAGCGCGAAA-3′	87 bp
MDM2 RP	356R	5′-GGTTAGCACCATCAGTAGGTACA-3′	
MDM2 Probe	300	5′-AGGAGCAGGCAAATGTGCAATACCAACAT-3′	
HPRT FP	537F	5′-GGCAGTATAATCCAAAGATGGTCAA-3′	80 bp
HPRT RP	616R	5′-GTCTGGCTTATATCCAACACTTCGT-3′	
HPRT Probe	567	5′-CAAGCTTGCTGGTGAAAAGGACCCC-3′	
TBP FP	645F	5′-GCCCGAAACGCCGAATAT-3′	73 bp
TBP RP	717R	5′-CCGTGGTTCGTGGCTCTCT-3′	
TBP Probe	664	5′-ATCCCAAGCGGTTTGCTGCGG-3′	
PSA FP	188F	5′-GTCTGCGGCGGTGTTCTG-3′	89 bp
PSA RP	276R	5′-TGCCGACCCAGCAAGATC-3′	
PSA Probe	226	5′-CACAGCTGCCCACTGCATCAGGA-3′	

FISH

For FISH analysis a PathVysion HER-2/*neu* DNA probe kit (Vysis, Inc., Downer's Grove, IL) was used according to the manufacturer's recommendation. Signals from 100 to 150 tumor cell nuclei were counted using confocal laser-scanning microscopy (Zeiss LSM 410). According to published criteria,[16] gene amplification was detected if the ratio of locus-specific signals to centromeric signals per cell was at least three in >10% of tumor cells, or tight clusters of >10 locus-specific signals occurred in multiple cells.

Immunohistochemistry

HER-2/neu protein expression was assessed on paraffin-embedded tissue samples using the anti-c-erbB-2 antibody (DAKO, Glostrup, Denmark) with a DAKO ChemMate detection kit according to the manufacturer's recommendation. To ensure the sensitivity of the reaction in all cases, immunoreactive tissue of an invasive ductal breast carcinoma with known overexpression of c-*erbB-2* was used as positive control. HER-2/neu protein overexpression was evaluated using a light microscope according to a score system as recommended by the DAKO HercepTest. Briefly, no staining at all or membrane staining in <10% of the tumor cells were scored as 0; a barely perceptible membrane staining in >10% of the tumor cells was scored as 1+; a weak-to-moderate staining of the entire membrane in >10% of the tumor cells was given a score of 2+; and a strong staining of the entire membrane in >10% of the tumor cells was scored as 3+.

Results

RNA Extraction from Formalin-Fixed, Paraffin-Embedded Tissues

As a first step toward the establishment of a quantitative gene expression methodology applicable to formalin-fixed, paraffin-embedded tissue, we examined RNA extracted by five different procedures from single routine paraffin sections by real-time TaqMan RT-PCR analysis. The expression of five cancer-relevant genes, *EGF-R*, *HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, and *MDM2* with mRNA half-lives ranging from 2 to 10 hours (unpublished results) was investigated.[17] All primers and hybridization probes were designed to span an intron to exclude annealing to genomic DNA and amplicon sizes were kept small (66 to 93 bp; see Table 1). *PGK* as well as *HPRT* were included as housekeeping gene controls to correct for variations in the degree of RNA degradation and efficiencies of RNA extraction and reverse transcription. After optimization of the experimental conditions for the seven primer/probe combinations, controls were performed to demonstrate the specificity of the RT-PCR reaction. No signals were obtained with genomic DNA or when reverse transcriptase or RNA were omitted (data not shown). Reproducibility and sensitivity of the extrac-

Table 2. Comparison of Different RNA Extraction Procedures

RNA extraction method*	Ct (p21/WAF1/Cip1)		
	Assay 1	Assay 2	Assay 3
Acidic guanidinium thiocyanate/phenol-chloroform[13]	24.59	25.02	26.02
Proteinase K digestion (60°C)	23.2	23.25	23.08
Proteinase K digestion (42°C)	26.39	26.62	26.35
Oligo d(T)-coupled magnetic beads	30.67	30.47	31.12
Guanidinium thiocyanate lysis/silica matrix	34.55	36.38	35.76

*RNA was extracted from single 5-μm adjacent sections of formalin-fixed, paraffin-embedded liver tissue using five different extraction procedures in three independent experiments as described in Materials and Methods. Shown are the mean Ct values of duplicate measurements for each of the three RNA preparations. Standard deviations on all points were below 0.3 and are omitted.

tion procedure and subsequent quantitative real-time RT-PCR amplification of the seven target sequences was assessed by different parameters of the TaqMan assay, such as low Ct and high ΔRn values (data not shown). Proteinase K digestion at high temperature followed by organic extraction (described in Materials and Methods) yielded the highest RNA amounts and the most reproducible results as compared to RNA extraction using Proteinase K digestion at 42°C, acidic guanidinium thiocyanate-phenol chloroform,[13] oligo d(T) coupled magnetic beads,[14] or guanidinium thiocyanate lysis combined with a silica-matrix spin technology[15] as shown representatively for the p21/WAF1/Cip1 Ct values (Table 2).

Quantitative Gene Expression Analysis in Matched Frozen and Formalin-Fixed, Paraffin-Embedded Tissue

To investigate gene expression in matched frozen and formalin-fixed, paraffin-embedded tissue, we used nude mice subcutaneous tumor xenografts of human colon adenocarcinoma HT29 (Figure 1A) and epidermoid carcinoma A431 cell lines because of their homogeneous histological characteristics. Isolation of RNA from 5-μm sections of A431 and HT29 tumors yielded only slightly more material for frozen than for formalin-fixed, paraffin-embedded tissue material. On serial dilutions of both preparations, the RNA was reverse-transcribed and real-time amplification of *EGF-R*, *HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, *MDM2*, *PGK*, and *HPRT* sequences was performed. In all cases, there was a strong linear correlation between the number of thermal cycles required to generate a significant fluorescent signal above background and the log of the input cDNA amount ($R^2 \geq 0.99$). Quantitative measurements could be made over an extremely wide range of target concentration (16 pg to 50, 000 pg). Even more importantly, when the resulting Ct values were plotted against the log of the initial template amount and subjected to linear regression analysis, the amplification efficiencies were found to be very similar in formalin-fixed, paraffin-embedded and frozen tissue xenografts as representatively shown for the *EGF-R* gene in HT29 tumors (Figure 1, B and C).

We then calculated relative amounts of *EGF-R*, *HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, and *MDM2* mRNAs in relation to *PGK* as a housekeeping gene in formalin-fixed, paraffin-embedded tumor xenograft tissue and com-

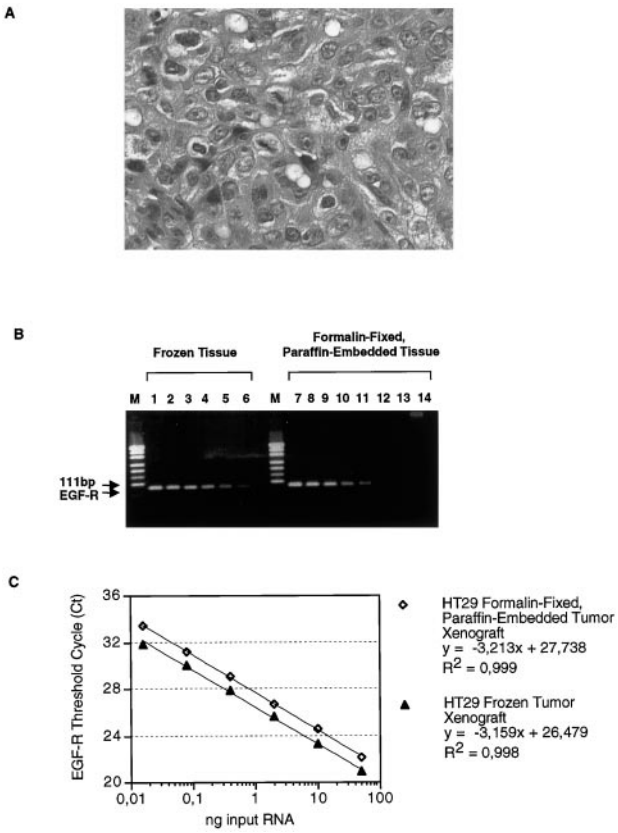

Figure 1. Quantitative gene expression analysis of *EGF-R* mRNA measured by real-time TaqMan QRT-PCR in matching frozen and formalin-fixed, paraffin-embedded HT29 human tumor xenografts. **A:** Five-μm section of a formalin-fixed, paraffin-embedded HT29 tumor xenograft stained with H&E, demonstrating the homogenous tumor histology. Original magnification, ×400. **B:** Serial dilutions of total RNA extracted from matching frozen and formalin-fixed, paraffin-embedded tissue samples from a HT29 tumor xenograft were subjected to 45 cycles of real-time TaqMan QRT-PCR of the *EGF-R* sequence. Shown are the PCR products on a 3% agarose gel after electrophoresis and ethidium bromide staining. **Lane M:** *MSP I*-digested pUC molecular weight DNA. **Lanes 1** and **7:** 50 ng RNA. **Lanes 2** and **8:** 10 ng RNA. **Lanes 3** and **9:** 2 ng RNA. **Lanes 4** and **10:** 0.4 ng RNA. **Lanes 5** and **11:** 0.08 ng RNA. **Lanes 6** and **12:** 16 pg RNA. No signals were generated using no-template control reactions (**Lane 13**) or genomic DNA (**Lane 14**). The **arrow** indicates the 93-bp *EGF-R* amplification product. **C:** The logarithm of the input RNA amount of the same samples is plotted *versus* the threshold cycle (Ct) monitored during real-time TaqMan QRT-PCR. Amplification efficiency of the *EGF-R* gene in matching frozen and formalin-fixed, paraffin-embedded tissue samples from HT29 tumor xenografts is comparable, as indicated by similar slopes of the regression lines. All points represent the mean of duplicate PCR amplifications, but error bars are too small to be visible.

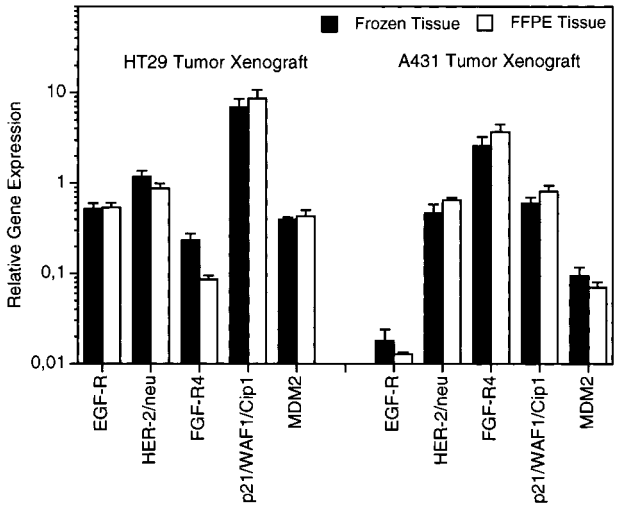

Figure 2. Quantitative determination of gene expression measured by real-time TaqMan QRT-PCR in matching frozen and formalin-fixed, paraffin-embedded HT29 and A431 human tumor xenografts. Levels of *EGF-R*, *HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, *MDM2*, and *PGK* mRNAs were determined by QRT-PCR and all measurements are shown relative to the expression levels of the *PGK* housekeeping gene. Results shown are the mean of three independent RNA isolations from single consecutive 5-μm sections ±SEM (n = 3).

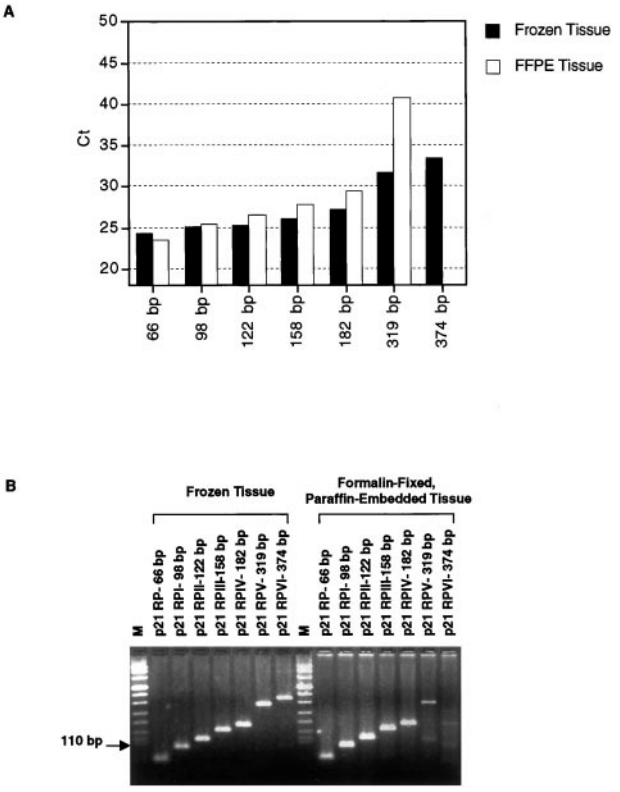

Figure 3. Effect of amplicon size on quantitative gene expression analysis by TaqMan QRT-PCR in matched frozen and formalin-fixed, paraffin-embedded tissue samples from A431 xenograft tumors. Seven primer/probe pairs for QRT-PCR were tested that amplify 66-, 98-, 122-, 158-, 182-, 319-, and 374-base-long portions of *p21/WAF1/Cip1* mRNA. The same forward primer and TaqMan probe were used in each case. **A:** Absolute Ct values for the seven primer pair combinations. Best results are obtained in FFPE tissue with QRT-PCR primer pairs spanning fragments <100 bp. Shown are the results of duplicate measurements but the standard deviations are too small to be seen. **B:** PCR products on a 3% agarose gel after electrophoresis and ethidium bromide staining. **Lane M:** Molecular Weight Marker VIII (Roche Molecular Biochemicals). No PCR product is generated from FFPE A431 tumor xenograft tissue with fragments >374 bp.

pared the results with those from the corresponding frozen xenograft sections. As shown in Figure 2, there was no significant difference between gene expression levels obtained with frozen or formalin-fixed, paraffin-embedded xenograft sections. Subsequently, the comparability of gene expression analysis results from matched frozen and formalin-fixed, paraffin-embedded material was confirmed with colorectal carcinoma samples and lymph node metastases from four patients, further demonstrating the value of the procedure for clinical research (data not shown).

To determine the optimal amplicon size for measuring mRNA levels in FFPE tissues, we designed sets of primer pairs spanning 66 to 374 bases for QRT-PCR amplification of *p21/WAF1/Cip1* mRNA and compared the resulting absolute Ct values of matched frozen and FFPE A431 tumor xenograft cDNA preparations. Figure 3, A and B, shows that amplicon sizes up to 122 bp gave the best results for FFPE tissue as indicated by low Ct values, whereas no cDNA product was obtained with primers that were 374 bases apart. When template from matching frozen control tissue was used under the same reaction conditions, PCR products up to 374 bases were easily detectable.

Effect of Delayed Formalin Fixation

To address parameters of the formalin fixation procedure, which depending on the clinical setting may influence gene expression measurements, we asked whether fixation grade can affect mRNA quantitation. Because tissue infiltration by the fixative is a slow process, extensive degradation of RNA may occur in the center of big specimens. We therefore performed quantitative gene expression analysis in big (>7 cm) tissue samples with decreasing grades of fixation toward the center. Because of their morphological homogeneity, we analyzed liver and uterus with leiomyoma tissues that had been fixed in formalin for 20 hours. Sequential sections of 1 cm were cut to a depth of 3 to 6 cm, and the blocks were paraffin-embedded using standard procedures. From every single cm of this tissue, RNA extraction was performed to measure gene expression of the five markers and two control genes to analyze whether differences in fixation might influence RNA expression measurements. The results of the relative gene expression measurements were found not to be significantly different with the exception of p21/WAF1/Cip1, which was up-regulated nearly 11-fold in leiomyoma tissue as compared to the adjacent myometrium. The latter observation was confirmed by immunohistochemical analysis (data not shown) suggesting that this finding was not caused by an intrinsic methodological artifact. Relative measurements and absolute Ct values for the *PGK* and *HPRT* housekeeping genes are shown in Figure 4; A, B, and C.

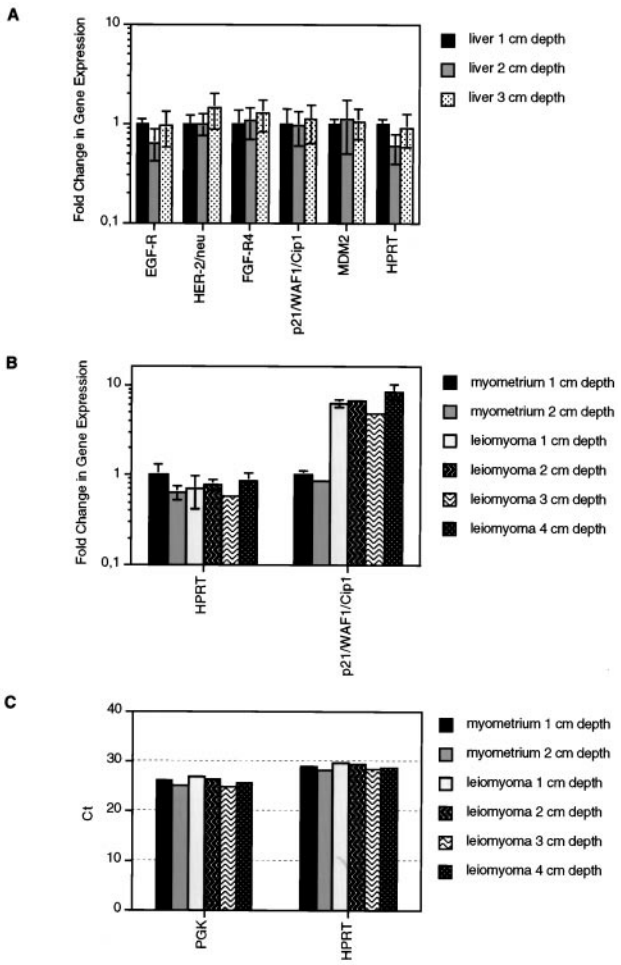

Figure 4. Influence of the formalin fixation procedure on quantitative gene expression analysis by TaqMan QRT-PCR. Liver and uterus with leiomyoma tissue samples that had been fixed for 20 hours were cut in sequential sections of 1 cm to a depth of 3 cm (liver) or 6 cm (uterus with leiomyoma). Quantitation of *EGF-R*, *HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, *MDM2*, and *HPRT* (liver) and *p21/WAF1/Cip1*and *HPRT* (uterus and leiomyoma) was determined relative to *PGK* as a housekeeping gene in single 5-μm sections of the sequential blocks. Change in gene expression of the six different genes is shown relative to a 5-μm section at 1-cm depth for liver (**A**) and uterus with myometrium and leiomyoma (**B**). Shown are the results of two different measurements, each run in duplicate ±SEM. (**C**) Absolute Ct values for PGK and HPRT that were used for quantitation of relative expression of the mRNAs.

Specificity of Real-Time RT-PCR Amplification in Formalin-Fixed, Paraffin-Embedded Tissues

To further demonstrate the specificity of the approach, prostate-specific antigen (*PSA*) mRNA amounts were measured in a formalin-fixed, paraffin-embedded prostate cancer specimen and in liver, myometrium, and leiomyoma tissues. As expected, the measurement of *PSA* mRNA resulted only in a signal with RNA isolated from the prostate cancer specimen and not with RNA isolated from the control tissues (Figure 5B). In contrast, the *TBP* mRNA that was determined as a positive control was readily detectable in all four tissues examined as shown in the amplification plots in Figure 5A and the corresponding analytical agarose gel in Figure 5C.

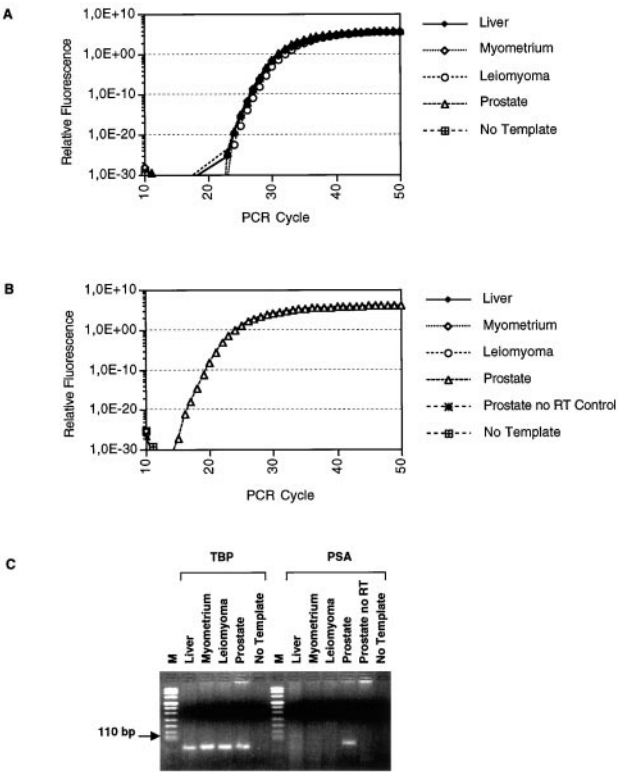

Figure 5. Specificity of the TaqMan QRT-PCR amplification. **A:** Real-time RT-PCR amplification plot of *TBP* mRNA measured in formalin-fixed, paraffin-embedded liver, myometrium and leiomyoma tissues, and a prostate cancer specimen. mRNA-specific signals are detectable in all four tissue types. **B:** Real-time RT-PCR amplification plot of *PSA* mRNA in the same tissues as indicated above, showing the specific detection of the PSA mRNA transcript only in the prostate cancer specimen. **C:** The same PCR products subjected to 3% agarose gel electrophoresis and ethidium bromide staining. **Lane M:** Molecular Weight Marker VIII (Roche Molecular Biochemicals). The **arrow** indicates the 110-bp fragment of the Molecular Weight Marker VIII.

Real-Time RT-PCR after Laser Microdissection from Formalin-Fixed, Paraffin-Embedded Tissue Sections

Laser-assisted microdissection has emerged as an important technique for the analysis of morphologically defined areas of tissue sections. To examine the applicability of our approach to such small tissue samples, we microdissected cell clusters consisting of ~10,000, ~1,000, 100, and 50 tumor cells from H&E-stained formalin-fixed, paraffin-embedded A431 and HT29 xenograft sections and determined gene expression levels in relation to a complete 5-μm section containing ~10^6 cells. Three independent experiments involving separate cell picking, RNA extraction, and reverse transcription were performed and expression of the seven different transcripts were examined from the same reverse transcription reaction. As shown in Figure 6, mRNA level determinations were comparable within the range of 10^6 to 100 cells for all six genes analyzed, demonstrating accuracy and reproducibility of the procedure. For *FGF-R4* and *MDM2* genes, expression in 50 microdissected cells from HT29 xenografts was at the borderline of detection, whereas the remaining genes of our panel yielded values comparable to all other preparations.

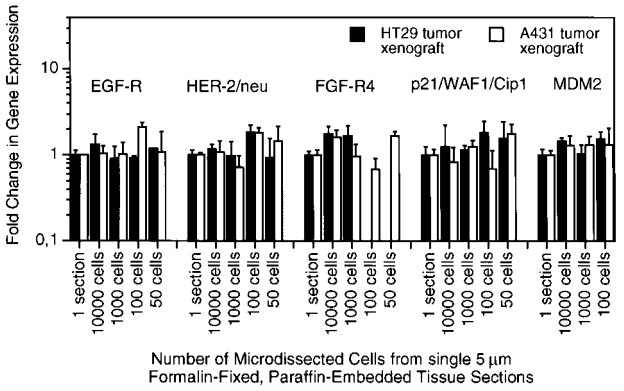

Figure 6. Quantitative gene expression analysis by TaqMan QRT-PCR after laser-assisted microdissection from formalin-fixed, paraffin-embedded tissues samples. Relative fold change in gene expression in microdissected A431 and HT29 tumor xenografts was calculated relative to gene expression in a complete 5-μm section. Defined numbers of cells (n_{cell} = ~10000, ~1000, 100, and 50) were microdissected from 5-μm H&E-stained formalin-fixed, paraffin-embedded sections, followed by quantitation of *EGF-R*, *HER-2/neu*, *FGF-R4*, *p21/WAF1/Cip1*, *MDM2*, and *PGK* mRNAs by real-time RT-PCR amplification. Expression of the different transcripts was examined from the same reverse transcription reaction and were determined relative to *PGK* as a housekeeping gene. Shown are the results of three independent cell picking, RNA isolation, and QRT-PCR amplifications ±SEM relative to a complete 5-μm section.

Quantitative HER-2/neu mRNA Analysis in Microdissected Archival Tumor Tissue

To further apply our approach in a clinically relevant context, we measured HER-2/*neu* gene expression in 54 laser-microdissected formalin-fixed, paraffin-embedded samples of 26 patients with esophageal adenocarcinoma. Real-time TaqMan RT-PCR performed on RNA extracted from laser-microdissected tumors (n = 26) revealed relative HER-2/*neu* mRNA expression levels from 0.27 to 83.57, whereas the surrounding normal squamous epithelium (n = 17) and gastric mucosa (n = 11) showed expression levels in a range of 0.26 to 1.7 (mean, 0.88 ± 0.38) and 0.36 to 2.7 (mean, 1.03 ± 0.59), respectively. Numerous studies show that amplification of the HER-2/*neu* gene and overexpression of the HER-2/neu protein closely parallel HER-2/*neu* mRNA overexpression.[18] We therefore compared the values generated by real-time QRT-PCR to corresponding DNA and protein data obtained by FISH and immunohistochemistry conducted on adjacent serial sections of the same tissue blocks. Among the 26 adenocarcinoma samples tested, 10 cases (38%) were found to have both an amplified HER-2/*neu* gene and to overexpress the HER-2/neu protein (except one case, no. 5, which displayed very weak HER-2/neu protein expression despite high-level HER-2/*neu* gene amplification). All of these 10 cases show significantly higher levels of HER-2/*neu* mRNA in the tumor than in the surrounding normal tissue (Table 3). The other 16 cases without HER-2/*neu* gene amplification showed relative HER-2/*neu* mRNA expression levels in the range of 0.27 to 1.1 (data not shown).

Discussion

With the completion of the human genome project in reach,[1] a need is building for experimental techniques that permit large scale retrospective studies toward the assessment of the diagnostic, prognostic, and therapeutic significance of newly discovered genes. Formalin-fixed, paraffin-embedded resected tissue samples have been collected throughout decades of routine histopathological examination and are a valuable resource for diagnostic and investigative studies: given the wide availability of the paraffin-embedded tissue blocks along with the clinical histories of the patients, both common and rare diseases can be studied retrospectively; furthermore, the cell architecture and morphology are excellently preserved, which is a prerequisite for exact histopathological diagnosis.[19]

The aim of this study was to develop an experimental procedure that would allow an accurate, reproducible, quantitative, high-throughput analysis of specific mRNA levels in standard paraffin-embedded pathology specimens.

Previous studies reporting molecular analyses of RNA from formalin-fixed, paraffin-embedded tissue present findings of a mostly qualitative nature, such as the detec-

Table 3. Comparison of TaqMan QRT-PCR, FISH, and Immunohistochemistry Analyses of Formalin-Fixed, Paraffin-Embedded Barrett's Adenocarcinoma

Case no.*	HER-2/neu FISH†	HER-2/neu IHC‡	*HER-2/neu* mRNA in microdissected tumor lesions§	*HER-2/neu* mRNA in microdissected corresponding normal tissue§
1	Amplification	3+	4.06 ± 0.42	1.16 ± 0.08
2	Amplification	3+	5.42 ± 0.92	0.59 ± 0.2
3	Amplification	3+	5.65 ± 0.34	0.57 ± 0.09
4	Amplification	3+	7.09 ± 0.52	0.97 ± 0.23
5	Amplification	1+	14.37 ± 1.24	1.05 ± 0.05
6	Amplification	3+	14.37 ± 1.24	1.05 ± 0.05
7	Amplification	3+	16.97 ± 0.59	0.56 ± 0.19
8	Amplification	3+	29.96 ± 3.22	0.68 ± 0.21
9	Amplification	3+	37.01 ± 1.87	1.72 ± 0.08
10	Amplification	3+	83.57 ± 7.52	1.11 ± 0.1

*Twenty-six cases of adenocarcinomas were analyzed by the three different methods, but only the cases with amplified *HER-2/neu* gene and HER-2/neu protein overexpression are shown.
†HER-2/neu status was assessed by FISH depending on the most prevalent cell population.
‡HER-2/neu protein staining was scored according to the score system recommended by the DAKO Hercep Test.
§Relative *HER-2/neu* gene expression values (*HER-2/neu*/PGK) ± SEM. Shown are the results of three independent measurements.

tion of viral nucleic acids in human tissues, or identification of tumor-specific products of chromosomal translocations and results of gene mutations.[20–22] Because of the lack of methodologies that allow accurate and simple quantitative measurements, there are only very few studies that report attempts of quantitative gene expression analysis in archival tissues to date.[23] A variety of approaches routinely used to assess the expression of specific genes in cells or tissues, such as Northern blot-, RNase protection-, S1 nuclease-, or *in situ* hybridization analysis have considerable drawbacks when applied to formalin-fixed, paraffin-embedded tissue sections: either the fragmented nature of the RNA precludes the application of these techniques or they may not be performed with small tissue samples, clinical biopsies, and microdissected cell clusters. Moreover, the sensitivity and accuracy of some of these techniques is limited. In contrast, the recently developed real-time quantitative RT-PCR is an easy, versatile, sensitive as well as accurate and precise method for the study of gene expression.[9,10] Real-time systems are capable of detecting PCR products as they accumulate during amplification, and the reactions are characterized by the point during cycling when PCR amplification is still in the exponential phase, thus enabling precise quantitation of RNA throughout a wide dynamic range. As little as 10 copies of a specific transcript can be detected and quantified. Moreover, the assay is compatible with high-throughput analysis, allowing 96 samples to be analyzed in only 2 hours.

We used real-time QRT-PCR based on TaqMan methodology to quantitatively analyze gene expression in routinely processed formalin-fixed, paraffin-embedded tissues and demonstrate that gene expression measurements can be reliably and accurately conducted in such tissues. A prerequisite for our gene expression studies in formalin-fixed tissue samples was the establishment of a reliable and reproducible microscale RNA extraction method in conjunction with reverse transcription that would provide an optimal cDNA as template for real-time PCR amplification. To date, various methods have been used for the isolation of RNA from archival formalin-fixed tissues and we compared five of the most commonly used ones in conjunction with TaqMan RT-PCR as quality control.[6,13–15] Our experiments showed that proteinase K digestion followed by organic extraction of the RNA yields the best results in terms of both reliability and sensitivity of the subsequent QRT-PCR (Table 2). It seemed that the protease was capable of efficiently degrading proteins that were covalently cross-linked with each other and the RNA, thereby allowing more efficient RNA extraction than chaotropic agents. In line with observations reported by Masuda and colleagues,[8] incubation of the tissue extracts or the already extracted RNA at high temperature (60 to 70°C) proved to be another important parameter, possibly by reversing the methylol additions induced by the formalin fixation and thereby improving reverse transcription efficiency. Most importantly, amplification protocols that involved only small RNA target sequences proved to be most successful presumably because of the significantly reduced risk of cross-link occurrence in the region bordered by the PCR primers (Figure 3, A and B) confirming an observation that previously has been made by Goldsworthy and colleagues.[24]

Using the proteinase K digestion method, we did not find significant differences in the amount of extracted RNA between fixed and fresh-frozen 5-μm sections (Figure 1, B and C). Indeed, our experiments with matched frozen and formalin-fixed, paraffin-embedded tissue from human tumor cell-line xenograft model tissues demonstrated that gene expression level measurements were in a comparable range (Figure 2). Regardless of the quantity of extractable RNA from formalin-fixed tissues and assuming that the degree of cross-linkage induced by the formalin fixation is similar in all mRNAs of a given sample, the use of a housekeeping gene as an internal reference standard is of great importance. This allows the accurate control of cDNA amounts as well as the quality of the extraction and reverse transcription steps thereby ensuring reliable mRNA quantitation.

Previous studies concluded that RNA in formalin-fixed, paraffin-embedded tissues undergoes significant enzymatic degradation[6,24–26] because of the different length of time until the specimens are fixed in formalin after surgical removal or because of variable influences during the processing of the tissues until complete fixation and embedding is achieved. Interestingly, our investigation of parameters relevant in this context indicate that within a period of 20 hours, the extent of formalin fixation has no major influence on the suitability of RNA from paraffin-embedded tissues for real-time QRT-PCR analysis (Figure 4), whereas we cannot rule out the possibility that the degree of RNA degradation differs under certain fixation conditions and may vary from tissue to tissue. However, even in a morphologically homogenous, RNase-rich tissue such as liver, transcript levels of the seven genes with mRNA half-lives ranging from 2 to 10 hours were not significantly altered in the different tissue layers (Figure 4A). We therefore conclude that although the RNA may undergo degradation, the resulting fragments are still large enough to be detected by TaqMan QRT-PCR. Seemingly, our findings are in disagreement with other reports; however, the amplicon sizes we choose were much smaller than those used in other studies.[7,20,21] Moreover we only used resected surgical specimens that had been fixed within 1 to 2 hours after removal and had been subject to a formalin fixation duration of a maximum of 72 hours. Until now, we successfully extracted and analyzed RNA from >250 formalin-fixed, paraffin-embedded tumor tissue blocks including some >20 years old. In all cases, we were able to extract amplifiable RNA with HPRT and PGK as control mRNA targets, suggesting that the time embedded in paraffin did not have an effect on the RNA. It should be noted, nonetheless, that the paraffin blocks were always sealed after preparation of slides, thus minimizing the risk of oxidization of tissue.

A procedure for reliable quantitative measurements of gene expression in archival tumor tissue is one important aspect when addressing basic questions regarding specific disease mechanisms; another critical point refers to the accurate access of cells to be examined.[27] To generate contamination-free, homogenous tumor cell popu-

lations, laser-microdissection has been introduced and is now generally accepted as a powerful tool to dissect morphologically identified cell populations.[11,12] Our laser microdissection studies with defined numbers of cells as starting material for QRT-PCR showed that quantitative gene expression measurements can be performed in as few as 50 microdissected cells from formalin-fixed, paraffin-embedded tissue sections (Figure 6). To our knowledge, this is the first report demonstrating that relative mRNA transcript levels can be reliably determined from such small cell numbers. Schütze and colleagues[28] reported single-cell RT-PCR from archival tissue however they used a nested RT-PCR approach to qualitatively detect c-Ki-ras2 mRNA mutations in colon adenocarcinoma. In another recent publication, Fink and colleagues[27] applied QRT-PCR to cell clusters containing 10 to 15 cells, but they used fresh-frozen tissue for their analyses. Fifty cells were the lowest number of cells used in the present study, however, when amplifying highly abundant mRNA transcripts, we even succeeded in performing single-cell real-time QRT-PCR from formalin-fixed, paraffin-embedded tissue (data not shown).

To further prove the clinical usefulness of our procedure, we choose HER-2/*neu* as the analyte prototype for validation. Overexpression and amplification of the HER-2/*neu* gene is known to be involved in many human cancers, including breast, ovarian, and gastrointestinal carcinoma and has been shown to correlate with tumor grade, tumor size, and disease progression.[18] Among the gastrointestinal cancers, esophageal Barrett's adenocarcinoma exhibits the most rapidly increasing incidence and ~20 to 60% of these adenocarcinomas show HER-2/*neu* gene amplification or overexpression of the HER-2/neu protein.[29–31] The results of our investigation indicate that among the 26 adenocarcinoma tested, 10 cases (38%) have both an amplified HER-2/*neu* gene and/or overexpress HER-2/neu protein and have significantly higher relative HER-2/*neu* transcript levels in tumor lesions than in the adjacent normal tissue (Table 3). The relative HER-2/*neu* expression ranges from 0.27 to 83.57 in tumor lesions as compared to only 0.26 to 1.7 in normal squamous epithelium, and 0.36 to 2.7 in gastric mucosa. Overall, HER-2/*neu* status assessed by FISH analysis and immunohistochemistry closely correlated with HER-2/*neu* gene overexpression measured by TaqMan QRT-PCR. Our findings in terms of the frequency of HER-2/*neu* amplification in Barrett's adenocarcinoma were in good agreement with published data.[29–31] As it is well established that amplification of the HER-2/*neu* gene and overexpression of the HER-2/neu protein closely parallel HER-2/*neu* mRNA overexpression,[18] these data represent a validation of our approach and suggest that it should be broadly and generally applicable to quantitative gene expression analysis in archival tissue.

In conclusion, we report the first application of combined QRT-PCR and laser-assisted microdissection for the quantification of gene expression levels in archival formalin-fixed, paraffin-embedded resected tissue samples. We demonstrated that mRNA levels in formalin-fixed, paraffin-embedded tissues routinely prepared from surgery and fixed with buffered formalin can be reproducibly and precisely determined and that the values obtained are comparable to matched frozen specimens. Controlled laser microdissection studies using defined numbers of cells showed that mRNA quantitation can be reliably performed from as few as 50 cells. Crucial aspects for the success of our procedure are: 1) an RNA microscale extraction protocol that provides only minimally cross-linked RNA, thus improving greatly the efficiency and the success of reverse transcription and QRT-PCR; and 2) the selection of small target sequences in a range of 60 to 100 bp, enabling the detection of fragmented and degraded RNA. Taken together, the combination of QRT-PCR and microdissection technologies with a reproducible microscale RNA extraction procedure establishes a simple, quantitative and highly accurate high-throughput procedure that allows retrospective studies and correlation of gene expression with clinicopathological features of large series of archival paraffin-embedded tumor tissue.

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